Molecular Analysis of $\beta_2$-Adrenoceptor Coupling to $G_{\text{s}}$-, $G_{\text{i}}$-, and $G_{\text{q}}$-Proteins

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ABSTRACT
The $\beta_2$-adrenoceptor ($\beta_2$AR) couples to the G-protein $G_{\text{s}}$ to activate adenylyl cyclase. Intriguingly, several studies have demonstrated that the $\beta_2$AR can also interact with G-proteins of the $G_{\text{i}}$- and $G_{\text{q}}$-family. To assess the efficiency of $G_{\text{i}}$- and $G_{\text{q}}$-family. To assess the efficiency of $\beta_2$AR interaction with various G-protein $\alpha$-subunits ($G_{\text{x}}\alpha$), we expressed fusion proteins of the $\beta_2$AR with the long ($G_{\text{x}}\alpha_{\text{L}}$) and short ($G_{\text{x}}\alpha_{\text{S}}$) splice variants of $G_{\text{s}}$, the $G_{\alpha}$-proteins $G_{\alpha_{\text{q}}}$ and $G_{\alpha_{\text{l}}}$, and the $G_{\alpha}$-proteins $G_{\alpha_{\text{q}}}$ and $G_{\alpha_{\text{l}}}$ in Sf9 cells. Fusion proteins provide a rigorous approach for comparing the coupling of a given receptor to $G_{\alpha}$, because of the defined 1:1 stoichiometry of receptor and G-protein and the efficient coupling. Here, we show that the $\beta_2$AR couples to $G_{\text{s}}$, $G_{\text{i}}$, and $G_{\text{q}}$-proteins as assessed by ternary complex formation and ligand-regulated guanosine 5’-O-(3-thiotriphosphate) (GTP-$\gamma$S) binding. The combined analysis of ternary complex formation, GTP-$\gamma$S binding, agonist efficacies, and agonist potencies revealed substantial differences in the interaction of the $\beta_2$AR with the various classes of G-proteins. Comparison of the coupling of the $\beta_2$AR and formyl peptide receptor to $G_{\alpha_{\text{q}}}$ revealed receptor-specific differences in the kinetics of GTP-$\gamma$S binding. We also detected highly efficient stimulation of GTP-$\gamma$S dissociation from $G_{\alpha_{\text{q}}}$, but not from $G_{\alpha_{\text{s}}}$ and $G_{\alpha_{\text{l}}}$, by a $\beta_2$AR agonist. Moreover, we show that the 1:1 stoichiometry of receptor to G-protein in fusion proteins reflects the in vivo stoichiometry of receptor/G-protein coupling more closely than was previously assumed. Collectively, our data show 1) that the $\beta_2$AR couples differentially to $G_{\text{s}}$, $G_{\text{i}}$, and $G_{\text{q}}$-proteins, 2) that there is ligand-specific coupling of the $\beta_2$AR to G-proteins, 3) that receptor-specific G-protein conformational states may exist, and 4) that nucleotide dissociation is an important mechanism for G-protein deactivation.

The $\beta_2$-adrenoceptor ($\beta_2$AR) is a prototypical G-protein-coupled receptor that interacts with the stimulatory G-protein of adenylyl cyclase, $G_s$ (Gilman, 1987; Kobilka, 1992). Intriguingly, studies of intact cells, cell membranes, and purified proteins have shown that the $\beta_2$AR can also interact with $G_s$-proteins (Katada et al., 1982; Asano et al., 1984; Xiao et al., 1995, 1999; Daaka et al., 1997; Pavoine et al., 1999). In addition, the $\beta_2$AR can activate phospholipase C-$\beta$ via G-proteins of the $G_{\text{q}}$-family, e.g., $G_{\text{1q}}$, and $G_{\text{qL}}$ (Zhu et al., 1994; Offermanns and Simon, 1995; Wu et al., 1995). In recent studies (Seifert et al., 1998a,b; Wenzel-Seifert et al., 1998b), we analyzed the coupling of the $\beta_2$AR to $G_{\alpha}$ using fusion proteins. In fusion proteins, the receptor $C$ terminus is covalently linked to the N terminus of $G_{\alpha}$. Fusion ensures a defined 1:1 stoichiometry of receptor to G-protein and promotes efficient coupling without altering the fundamental properties of the signaling partners. The fusion protein approach has been successfully applied to various receptors and G-proteins (Seifert et al., 1999c; Milligan, 2000). With the fusion protein approach we could dissect subtle differences in the coupling of the $\beta_2$AR to $G_{\alpha_{\text{s}}}$ and $G_{\alpha_{\text{l}}}$ (Seifert et al., 1998b). In the latter study, we analyzed receptor-G-protein coupling by measuring ternary complex formation, i.e., the complex of agonist, receptor, and nucleotide-free G-protein displaying high agonist affinity, steady-state GTP hydrolysis, and adenylyl cyclase activation.

The goal of our present study was to quantitatively compare the coupling of the $\beta_2$AR to $G_{\text{s}}$, $G_{\text{i}}$, and $G_{\text{q}}$-proteins. To achieve this aim, we needed a system that ensures defined receptor-G-protein stoichiometry and efficient coupling.

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ABBREVIATIONS: $\beta_2$AR, $\beta_2$-adrenoceptor; $\beta_2$AR-$G_{\alpha_{\text{s}}}$, $G_{\alpha_{\text{s}}}$; $G_{\alpha_{\text{i}}}$, the short splice variant of $G_{\alpha}$; $G_{\alpha_{\text{qL}}}$, the long splice variant of $G_{\alpha}$, and $G_{\alpha_{\text{L}}}$, respectively; DHA, [3H]dihydroalprenolol; DCl, dichloroisoproterenol; DOB, dobutamine; EPH, (–)-ephedrine; FPR, formyl peptide receptor; FPR-$G_{\alpha_{\text{q}}}$, fusion protein consisting of the FPR and $G_{\alpha_{\text{q}}}$; GTP-$\gamma$S, guanosine 5’-O-(3-thiotriphosphate); $G_{\alpha_{\text{i}}}$, nonspecified G-protein $\alpha$-subunit; ISO, (–)-isoproterenol; ICI, ICI 118,55 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol); SAL, salbutamol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription.
Therefore, we constructed various β2AR-Gx fusion proteins and analyzed those proteins in Sf9 insect cells. To validate the results obtained with the fusion protein consisting of the β2AR and Gi\(_{12/3}\) (β2AR-Gi\(_{12/3}\)), we also coexpressed the β2AR with Gi\(_{12}\). Moreover, we compared β2AR-Gi\(_{12}\) coupling with forml peptide receptor (FPR)-Gi\(_{12}\). The FPR is a prototypical G-protein-coupled receptor (Gierschik et al., 1991; Wenzel-Seifert et al., 1998a, 1999). Here, we report differential coupling of the β2AR to G\(_{a16}\), G\(_{\gamma}\), and G\(_{\delta}\) proteins and differences in the coupling of the β2AR and FPR to G\(_{a}\) proteins.

**Experimental Procedures**

**Materials.** The cDNAs of Gi\(_{12}\) and Gi\(_{13}\) in pGEM-2 were kindly provided by Dr. R. Reed (Howard Hughes Medical Institute, Johns-Hopkins-University, Baltimore, MD) (Jones and Reed, 1987). The cDNA of Gi\(_{a16}\) in pCMV was a gift from Dr. D. Wu (Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY) (Amatruda et al., 1991). The cDNA of Ga in pVL1392 was kindly provided by Dr. E. M. Ross (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX) (Biddlecome et al., 1996). Recombinant baculovirus encoding Gi\(_{12}\) was kindly provided by Dr. A. G. Gilman (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX). Antibodies recognizing Gi\(_{13}\) (anti-Gi\(_{13}\) AS 86, C terminal) (Leopoldt et al., 1997), all Ga subunits (anti-Ga common, AS 266) (Leopoldt et al., 1997), Go (anti-Go16, AS 339) (Spicher et al., 1994), and Gi\(_{16}\) (anti-Gi\(_{16}\), AS 389) (Spicher et al., 1994) were generously provided by Drs. B. Nurnberg and G. Schultz (Institut für Pharmakologie und Toxikologie, Universität Ulm, Ulm, Germany). The Gi\(_{a16}\) baculovirus was kindly provided by Dr. R. Reed (Howard Hughes Medical Institute, Johns-Hopkins-University, Baltimore, MD) (Jones and Reed, 1987). Antibodies recognizing G\(_{a12/3}\) (anti-Ga12/3 AS 86, C terminal) (Leopoldt et al., 1997), all Ga subunits (anti-Ga common, AS 266) (Leopoldt et al., 1997), Go (anti-Go16, AS 339) (Spicher et al., 1994), and Gi\(_{16}\) (anti-Gi\(_{16}\), AS 389) (Spicher et al., 1994) were generously provided by Drs. B. Nurnberg and G. Schultz (Institut für Pharmakologie und Toxikologie, Universität Ulm, Ulm, Germany). The antibody recognizing G\(_{a12}\) was from Calbiochem (La Jolla, CA). [\(^{3}H\)](S)Guanosine 5′-O-(3-thiotriphosphate (GTP\(_{\gamma}\)S); 1000–1500 Ci/mmol) was from NEN Life Science Products (Boston, MA). \(^{3}H\)Dihydroliprenol (DHA; 85–90 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ). Unlabeled GTP-S and GDP were obtained from Roche Diagnostics (Indianapolis, IN). ICI 118,55 (erythroid-3-17(methyl-indan-4-yl)-3-isopropylaminobutan-2-ol) (ICI) was from Research Biochemicals International (Natick, MA). The M1 monoclonal antibody (detecting the FLAG epitope), (−)isopropenol (ISO), salbutamol (SAL), (−)ephedrine (EPH), and (−)alprenolol were from Sigma (St. Louis, MO). Dichloroisopropenol (DCI) was from Aldrich (Milwaukee, WI). All restriction enzymes, DNA polymerase I, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Glass fiber filters (GF/C) were from Schleicher & Schuell (Dassel, Germany). All other reagents were of the highest purity available and from standard suppliers.

**Construction of the cDNAs Encoding β2AR-Gx Fusion Proteins.** The cDNA of β2AR-Ga in pGEM-32 (Seifert et al., 1998a,b) was used as a template to amplify the C-terminal portions of the β2AR. In this construct the β2AR is tagged at the 5′ end with a DNA sequence encoding the cleavable signal peptide (S) from influenza hemagglutinin, which facilitates correct insertion of the receptor into the plasma membrane, followed by the FLAG epitope (F), which can be recognized by the M1 antibody. The C terminus of the receptor is tagged with a hexahistidine tag. Fusion of the β2AR with different Ga\(_{\delta}\) subunits was achieved by sequential overlap-extension polymerase chain reactions (PCR) using Pfu polymerase (Stratagene, La Jolla, CA). In PCR 1A, the DNA sequence of the C terminus of β2AR was amplified with pGEM3Z-SP-β2AR-Ga\(_{12}\), as a template by using a sense primer 5′ of the SacI site in the C terminus (sense SacI primer) and an antisense primer encoding the hexahistidine tag. The cDNAs of the four different Ga\(_{\delta}\) subunits were amplified in four different PCR reactions (PCR 1B\(_{1-4}\)) using pGEM-2-Ga\(_{12}\), pGEM-2-Ga\(_{16}\), pCMV-Ga\(_{16}\), and pVL 1392-Ga\(_{12}\), respectively, as templates. The sense primers annealed with the first 18 bp of the 5′ end of Ga and included the 18 bp of the hexahistidine tag in their 5′ extensions. The antisense primers encoded the last five amino acids of the C terminus of the Ga\(_{\delta}\), followed by the stop codon and an extra XbaI site for cloning purposes in the 3′-end extension. In the case of Ga\(_{16}\), a BamHI site instead of an XbaI was included in the 3′-end extension of the antisense primer. In PCR 2 the cDNA fragments from PCR 1A and PCR 1B\(_{1-4}\) were annealed and amplified using the sense SacI primer and the antisense primers of PCR 1B\(_{1-4}\). In this way, fragments encoding the C terminus of β2AR, a hexahistidine tag, and the Ga\(_{\delta}\) were followed by an XbaI or BamHI site was obtained. The fragment for β2AR-Ga\(_{16}\) was digested with EcoRV and XbaI and cloned into pGEM3Z-β2AR digested with SacI and SalI together with an oligonucleotide linker encoding (5′–3′) an XbaI site, a BamHI site, and a SalI site. The fragments for β2AR-Ga\(_{16}\) and β2AR-Ga\(_{12}\) were digested with EcoRV plus XbaI or EcoRV plus BamHI, respectively, and cloned into pGEM3Z-β2AR-Ga\(_{12}\) digested with EcoRV plus XbaI or EcoRV plus BamHI, respectively. PCR-generated DNA sequences were confirmed by enzymatic sequencing using Sequenase version 2.0 Sequencing kit (USB, Cleveland, OH). For cloning into the baculovirus expression vector pVL 1392, the cDNAs encoding β2AR-Ga\(_{12}\) fusion proteins in pGEM-3Z were digested with HindIII at the 5′-end of the SP region, blunted with DNA polymerase I (Klenow fragment), and then digested with XbaI or BamHI at the 3′-end of the Ga\(_{12}\). Digested fusion protein DNA sequences were then ligated into pVL 1392 that had been digested with BglII, blunted with Klenow fragment, and subsequently digested with XbaI or BamHI.

**Generation of Recombinant Baculoviruses and Cell Culture and Membrane Preparation.** Recombinant baculoviruses encoding the β2AR-Ga, fusion proteins were generated in Sf9 cells using the BaculoGOLD transfection kit (Pharmingen, San Diego, CA) according to the manufacturer’s instructions. After initial transfection, working virus stocks were generated by three sequential virus amplifications. Sf9 cells were cultured in 250-ml disposable Erlenmeyer flasks at 28°C under rotation at 125 rpm in SF 900 II medium (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (Gemini, Calabasa, CA) and 0.1 mg/ml gentamicin (Roche Diagnostics). Cells were maintained at a density of 0.5 to 6.0 × 10^6 cells/ml. For infection, cells were sedimented by centrifugation and suspended in fresh medium. Cells were seeded at 3.0 × 10^6 cells and infected with a 1:100 dilutions of high-titer baculovirus stocks encoding β2AR-Ga\(_{12}\) fusion proteins or nonfused β2AR plus Ga\(_{12}\). Except for the experiments shown in Fig. 5, all cultures were also coinfected with a baculovirus encoding β2AR-Ga\(_{12}\) subunits at a 1:100 dilution. Cells were cultured for 48 h before membrane preparation.

Sf9 membranes were prepared as described (Seifert et al., 1998a), using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, and 10 μg/ml leupeptin as protease inhibitors.

**DHA Binding.** Before experiments, membranes were pelleted by a 15-min centrifugation at 4°C and 15,000g and resuspended in binding buffer (12.5 mM MgCl\(_2\), 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4). B\(_{max}\) values were determined by incubating Sf9 membranes (10–40 μg of protein/tube, depending on the specific expression level) with a single saturating concentration of DHA (10 nM). Nonspecific binding was determined in the presence of DHA (10 nM) plus 10 μM (−)alprenolol. Incubations were performed for 90 min at 25°C and shaking at 200 rpm. Competition binding experiments were carried out with 1 nM DHA in the presence of ISO at various concentrations with or without GTP-S (10 μM). Bound DHA was separated from free DHA by filtration through GF/C filters and washed three times with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting.

**GTPyS Binding and GTPyS Dissociation.** Membranes were thawed, pelleted by a 15-min centrifugation at 4°C and 15,000g, and resuspended in binding buffer. For GTPyS saturation binding studies, reaction mixtures (500 μl) contained membranes (10–81 μg of proteins).
protein (tube) in binding buffer supplemented with 0.05% (w/v) bovine serum albumen, 1 μM GDP, and 0.2 to 1 nM [35S]GTPγS plus unlabeled GTPγS at increasing concentrations to give the desired final ligand concentrations. Reaction mixtures additionally contained distilled water (control) and β2AR ligands at a saturating concentration (ISO, 10 μM; ICI, 1 μM). Incubations were performed at 25°C and shaking at 200 rpm for various periods, depending on the specific properties of the fusion protein. For time-course studies, S9 membranes were suspended in 1500 μl of binding buffer supplemented with 1 to 2 nM [35S]GTPγS plus 9 to 48 nM unlabeled GTPγS, 1 μM GDP, and distilled water (control) or β2AR ligands at a saturating concentration (ISO, 10 μM; ICI, 1 μM). Aliquots of 200 μl (containing 15–70 μg of protein) were taken at seven different time points. In the experiments shown in Fig. 5B, the [35S]GTPγS concentration was 0.4 nM. Assays were conducted in the absence of GDP or in the presence of GDP at 1 nM to 10 μM. In the experiments shown in Tables 2 and 3, reaction mixtures contained 0.4 nM [35S]GTPγS, 1 μM GDP, and different β2AR ligands at increasing concentrations. Nonspecific [35S]GTPγS binding was determined in the presence of 10 μM GTPγS and was less than 0.1% of total binding. Bound [35S]GTPγS was separated from free [35S]GTPγS by filtration through GF/C filters, followed by three washes with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting.

For assessing GTPγS dissociation, GTPγS (0.5 nM [35S]GTPγS plus 9.5 nM unlabeled GTPγS) was allowed to associate to membranes for 60 min in the absence of ligand. [35S]GTPγS dissociation was initiated by the addition of 20 μM unlabeled GTPγS in the absence or presence of GDP at 1 nM to 10 μM. In the experiments shown in Tables 2 and 3, reaction mixtures contained 0.4 nM [35S]GTPγS, 1 μM GDP, and different β2AR ligands at increasing concentrations. Nonspecific [35S]GTPγS binding was determined in the presence of 10 μM GTPγS and was less than 0.1% of total binding. Bound [35S]GTPγS was separated from free [35S]GTPγS by filtration through GF/C filters, followed by three washes with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting.

Results and Discussion

Analysis of the Expression of β2AR-Gxα Fusion Proteins by Immunoblotting. In our previous studies we had already documented that structurally intact β2AR-Gxα and β2AR-GxαS fusion proteins can be expressed in S9 cells (Seifert et al., 1998a,b). In immunoblots of membranes expressing β2AR-Gxα, β2AR-GxαS, β2AR-Gxαa, and β2AR-Gxαq, the M1 antibody recognized proteins with apparent molecular masses of ~90 kDa (Fig. 1A). These values correspond to the sum of the molecular mass of the β2AR (~50 kDa) and Gxα (~40 kDa). Note that there were no immunoreactive bands of lower molecular mass than ~90 kDa, indicating that β2AR-Gxα fusion proteins were not degraded by insect cell proteases. The anti-Gαq Ig also reacted with proteins of lower molecular mass than ~90 kDa. Because the immunoblot with the M1 antibody clearly demonstrated that fusion proteins were not degraded (see Fig. 1A), the results obtained with the anti-Gαq Ig suggest that the additional bands recognized by this antibody represent nonspecific reactions.

We also quantified the expression of nonfused Gxα in membranes coexpressing β2AR (2.3 pmol/mg) and Gxαa (Fig. 1B). By using membranes expressing β2AR-Gxα (100 μg of protein per lane) at a defined level (15 pmol/mg) as standard, we estimated that the expression level of nonfused Gxα is ~300 pmol/mg. Thus, the stoichiometry of the β2AR to Gxα in the coexpression system is ~1:100. This ratio is similar to the ratio of the β2AR to Gxα and of the FPR to Gxα expressed in S9 cells and most likely represents the in vivo β2AR/Gxα ratio (Ranasas and Insel, 1988; Gierschik et al., 1991; Seifert et al., 1998a; Wenzel-Seifert et al., 1998a, 1999).

We tried to detect β2AR-GxαS with anti-Gαa Ig (AS 339) (Spicher et al., 1994) and β2AR-Gxαq with anti-Gαq Ig (AS 369) (Spicher et al., 1994). However, the signals detected in the 90-kDa area were quite weak, and there were numerous nonspecific reactions with proteins of molecular mass lower than 90 kDa (data not shown). In addition, we could not convincingly detect β2AR-Gxα fusion proteins with the anti-Gxαmonomeric Ig (AS 266) (Leopoldt et al., 1997; data not shown). Evidently, the sensitivity of these antibodies is too low to detect the corresponding β2AR-Gxα fusion proteins at the expression levels achievable. This interpretation is supported by the fact that anti-Gxαmonomeric Ig detected nonfused GxαS expressed at ~300 pmol/mg quite well (Wenzel-Seifert et al., 1998a) but β2AR-Gxα fusion proteins are expressed at levels that are ~20 to 100 times lower than those of nonfused Gxα.
digested with various restriction enzymes (Fig. 1D). As expected, G1ox-, G1ox-, and G1ox cDNAs were digested by PstI. Because of its small size, the 83-bp fragment of G1ox is not visible. Digestion with BamHI gave the expected fragments with G1ox-, G1ox-, and G1ox-cDNA, and digestion with EcoRI resulted in cleavage of the cDNA of G1ox, and G1ox. Thus, the RT-PCR data confirm the specific expression of β2AR-G1ox fusion proteins in Sf9 cell membranes.

**Agonist-Competition Studies with β2AR-G1ox: Differential Ternary Complex Formation with Fusion Proteins.** One of the most stringent tests of receptor/G-protein coupling is the formation of the ternary complex, i.e., the complex consisting of agonist-occupied receptor and guanine nucleotide-free Gα (De Lean et al., 1980; Seifert et al., 1998b). This complex possesses high affinity for agonists and can be detected in radioligand binding studies in which unlabeled agonist competes with radiolabeled antagonist. Upon binding of GTP or its GTPase-resistant analog GTPγS, the ternary complex is disrupted, and the receptor converts into a state of low agonist affinity (De Lean et al., 1980; Seifert et al., 1998b).

In membranes expressing β2AR-G1ox and β2AR-G1ox plus β1γ-complex, 42.1 ± 3.4 and 51.2 ± 4.9% of the receptors, respectively, displayed high affinity for the full agonist ISO (Ki values, 0.7 ± 0.1 and 1.3 ± 0.3 nM, respectively) (Fig. 2, A and B). GTPγS converted the β2ARs into a single population of receptors displaying low agonist affinity (Ki values, 155 ± 19 and 182 ± 22 nM, respectively). These data are similar to our previous data on β2AR-G1ox fusion proteins expressed without the β1γ-complex (Seifert et al., 1998b) and demonstrate that mammalian βγ-complex is not required for efficient ternary complex formation in β2AR-G1ox fusion proteins. In membranes expressing β2AR-G1ox and β2AR-G1ox, there was no detectable high-affinity agonist bind-

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**Fig. 1.** Analysis of the expression of β2AR-G1ox fusion proteins and of nonfused G1ox in Sf9 membranes. A, Sf9 membranes (50 μg of protein/lane) expressing β2AR-G1ox (7.6 pmol/mg), β2AR-G1ox (6.0 pmol/mg), β2AR-G1ox (3.5 pmol/mg), or β2AR-G1ox (8.5 pmol/mg) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-FLAG Ig (M1 antibody). B, Sf9 membranes expressing β2AR-G1ox (15.0 pmol/mg, 100 μg of protein/lane), β2AR-G1ox (7.6 pmol/mg, 150 μg of protein/lane) or β2AR (2.3 pmol/mg) plus nonfused G1ox (micrograms of protein loaded per lane given below the lanes) were separated by SDS-PAGE, transferred to Immobilon-P transfer membranes, and probed with anti-G1ox Ig. C, Sf9 membranes expressing β2AR-G1ox (15.0 pmol/mg, 100 μg of protein/lane) or β2AR-G1ox (7.6 pmol/mg, 150 μg of protein/lane) or β2AR (2.3 pmol/mg) plus nonfused G1ox were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-G1ox Ig. For further details, see Experimental Procedures. Numbers on the left indicate molecular masses of marker proteins. Shown are the horseradish-peroxidase-reacted membranes of gels containing 10% (w/v) acrylamide. D, mRNA from Sf9 cells infected with β2AR-G1ox baculoviruses was isolated and reverse-transcribed as described under Experimental Procedures. The G1ox portions of fusion proteins were amplified by PCR and digested with PstI, BamHI, or EcoRI. Digested DNA was separated on gels containing 2% (w/v) agarose. DNA was stained with ethidium bromide. The left lanes of gels show the 100-bp DNA ladder. The broad calibration lanes represent the 100-, 500-, and 1000-bp standards. The scheme above the gels shows the relevant positions of the relevant restriction sites in G1ox.
ing (Fig. 2, C and F). G_{16}, conferred to 11.4 ± 2.9% of the β_{2}ARs the ability to bind agonist with high affinity (K_{i} 2.3 ± 0.4 nM) (Fig. 2E). The ternary complex formed with G_{16} was GTPγS sensitive. In membranes expressing β_{2}AR-G_{i6}, some high-affinity binding of ISO (14.9 ± 3.5%; K_{i}, 4.0 nM) could be detected as well, but this high-affinity agonist binding was GTPγS-insensitive (Fig. 2D). These data show that, with regard to ternary complex formation, the β_{2}AR couples much more efficiently to G_{s}- than to G_{i}- and G_{q}-proteins and that the β_{2}AR discriminates between different members of the G_{s}- and G_{q}-family. The GTPγS insensitivity of the ternary complex formed with G_{i3} reflects the inability of GTPγS to promote dissociation of this G-protein from the β_{2}AR. Indeed, GTPγS-insensitive ternary complex formation has been repeatedly observed (Szele and Pritchett, 1993; Gürdal et al., 1997; Seifert et al., 1998a) and points to permanent physical interaction of the receptor with the G-protein during the entire G-protein cycle (see also discussion below on receptor agonist-regulated GTPγS dissociation from G-proteins).

General Considerations for GTPγS Binding Studies with β_{2}AR-G_{xα} Fusion Proteins and Advantages and Disadvantages of the Sf9 Cell System. Receptors catalyze GDP release from G_{xα} and subsequently promote the binding of GTP or its hydrolysis-resistant analog GTPγS to the G-protein (Gilman, 1987; Gierschik et al., 1991; Iiri et al., 1998; Wenzel-Seifert et al., 1998a, 1999). Because the sensitivity of the GTPγS binding assay surpasses the sensitivity of the steady-state GTPase assay (Gierschik et al., 1991; Seifert et al., 1998a), the GTPγS binding assay has become the most widely used assay to monitor receptor-G-protein coupling directly at the G-protein level. The GTPγS binding assay allows for the quantitative comparison of the coupling of a given receptor to different G-proteins and of different receptors to a given G-protein because the measurement of GTPγS binding is independent of an effector system.

A unique property of fusion proteins is the 1:1 stoichiometry of receptor and G-protein (Seifert et al., 1999c; Milligan, 2000). If each receptor interacts only with its fused G_{xα} partner, the B_{max} values of ligand-regulated GTPγS binding and radioligand antagonist binding should be similar. Ligand-regulated GTPγS binding is the difference between maximum agonist-stimulated GTPγS binding and minimum GTPγS binding in the presence of an inverse agonist (Wenzel-Seifert et al., 1998a, 1999). The ratio of the B_{max} of ligand-regulated GTPγS binding and the B_{max} of radioligand antagonist binding is defined as the coupling factor and should be approximately 1 for fusion proteins if the receptor interacts only with its fused G_{xα} partner (Wenzel-Seifert et al., 1999).

To quantitate β_{2}AR coupling to insect cell G-proteins, we performed GTPγS saturation binding studies with Sf9 cell

Fig. 2. Competition by ISO of DHA binding in Sf9 membranes expressing β_{2}AR-G_{xα}. Effect of GTPγS. Competition by ISO of DHA binding in Sf9 membranes was performed as described under Experimental Procedures. Reaction mixtures contained Sf9 membranes (10–40 µg of protein/tube) expressing the fusion proteins identified in A to F at 2.1 to 18.6 pmol/mg, 1 nM DHA, and ISO at the concentration indicated on the abscissa. The DHA binding observed in the absence of competitor is referred to as basal binding and was set 100%. Reaction mixtures additionally contained distilled water (control) or GTPγS (10 μM). Data points shown are the means ± S.D. for three to five experiments performed in triplicate. Agonist competition curves were analyzed for best fit to single-site and two-site competitions.
membranes expressing nonfused β2AR. The B_{max} of ISO-stimulated GTPγS binding in Sf9 membranes expressing β2AR was extremely low. The coupling factor of 0.01 implies that only one G-protein per 100 expressed β2AR molecules was activated upon agonist stimulation (Table 1). The poor coupling of the β2AR to insect cell G-proteins underlines the feasibility of the Sf9 cell system for GTPγS binding studies.

However, it should be emphasized that, despite poor coupling of the β2AR to insect cell G-proteins, there is a high concentration of as yet poorly defined GTPγS binding sites in Sf9 cell membranes. This results in high basal GTPγS binding rates (Fig. 3). Although those GTPγS binding sites are irrelevant with respect to G-protein coupling of the β2AR (Table 1), these binding sites reduce, nonetheless, the sensitivity of the GTPγS binding assay. Grunewald et al. (1996) reported high basal GTPγS binding in Sf9 membranes, too. To eliminate this background GTPγS binding and to analyze GTPγS binding to the different G-proteins under comparable conditions, we focused our attention on ligand-regulated GTPγS binding to fusion proteins. The validity of this approach and the assumption that basal GTPγS binding is irrelevant to fusion proteins is substantiated by the finding that the coupling factor for most fusion proteins was, as expected, ~1 (Table 1). We realize that for determination of absolute K_{d} values for GTPγS binding to G-proteins, it would have been more appropriate not to subtract basal GTPγS binding values, but the considerable background (in particular for β2AR-Gi, and β2AR-Gs fusion proteins) prevented us from doing so. To take into consideration this limitation of our studies, we use the term apparent K_{d} value where appropriate. We already adopted the background subtraction approach to the analysis of GTPγS binding to G_{i}-proteins coupled to the FPR in the fused and nonfused state (Wenzel-Seifert et al., 1998a, 1999). Thus, our approach allows for relative comparison of apparent K_{d} values for GTPγS binding to different G-proteins coupled to the β2AR and to the same G-protein coupled to the β2AR and FPR.

**Time Course of GTPγS Binding to β2AR-Gxα13: Comparison with the FPR/Giα1 Pair and Possible Physiological Implications.** We studied the time course of GTPγS binding to β2ARGxα13 fusion proteins in the presence and absence of a saturating concentration of ISO. The rate of GTPγS association to G-proteins is determined by the rate of GDP release (Gilman, 1987; Higashijima et al., 1988, 1990). In membranes expressing β2AR-Gxα13, ISO decreased the apparent t_{1/2} of GTPγS association about 7-fold (Fig. 3A). However, GTPγS binding at late time points of the reaction was no longer stimulated by ISO. In our previous study we showed that the β2AR coupled to G_{sα1} possesses constitutive activity, i.e., even the agonist-free receptor can efficiently promote GDP release from G_{sα1} (Seifert et al., 1998b). Thus, we assumed that the lack of agonist effect on GTPγS binding to β2AR-Gxα13, at late time points of the binding reaction reflects the ability of the agonist-free β2AR to efficiently promote GTPγS binding to the G-protein. To validate this assumption, we studied the effect of the inverse agonist ICI on GTPγS binding. As expected, ICI substantially inhibited GTPγS binding to β2AR-Gxα13, particularly at late time points of the reaction (Fig. 3A).

ISO also accelerated GTPγS binding in membranes expressing β2AR-Gsα13, but in contrast to membranes expressing β2AR-Gxα13, a stimulatory effect of ISO was evident even at late time points of the binding reaction (Fig. 3B). We also noted that the t_{1/2} of ISO-stimulated GTPγS binding to G_{sα1} was about twice as high as for G_{xα1}. These data can be interpreted in that the agonist-occupied β2AR catalyzes GDP release from G_{sα1} with a slower rate than from G_{xα1} (Seifert et al., 1998b). ICI had only a minimal inhibitory effect on GTPγS binding to β2AR-Gxα13 (data not shown).

With respect to β2AR-Gxα13, β2AR-Gsα13, β2AR-Gqα13, and β2AR-Giα13, a significant stimulatory effect of ISO on GTPγS binding was detected at all time points of the reaction. In contrast to β2ARGsα13 fusion proteins, there was no significant (β2ARGxα13, β2ARGqα13, and β2ARGiα13) stimulation by ISO and only a small decrease (β2AR-Gsα13, β2AR-Gqα13) of the t_{1/2} of basal GTPγS association by ISO. In addition, the t_{1/2} values for ISO-stimulated GTPγS binding to membranes expressing β2AR-Gsα13 and β2AR-Gqα13 fusion proteins were at least 3-fold higher than for β2ARGxα13 fusion proteins. These data indicate that the β2AR promotes guanine nucleotide exchange at G_{s} and G_{q} proteins much more slowly than at G_{i} proteins.

The t_{1/2} of FPR agonist-stimulated GTPγS binding to fused and nonfused G_{xα1} is much lower (~5 min) (Wenzel-Seifert et al., 1998a, 1999) than the t_{1/2} of β2AR-stimulated GTPγS binding to fused G_{xα1} (~45 min) (Fig. 3C), indicating that the FPR promotes guanine nucleotide exchange at G_{xα1} much more rapidly than the β2AR. Based on these findings, one can assume that cellular responses mediated by the G_{s} and G_{q}.

### Table 1

<table>
<thead>
<tr>
<th>Construct</th>
<th>Apparent Kd of Agonist-Stimulated GTPγS Binding</th>
<th>Apparent Kd of Agonist-Inhibited GTPγS Binding</th>
<th>Coupling Factor</th>
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<td>β2AR</td>
<td>2.4 ± 2.3</td>
<td>—</td>
<td>—</td>
</tr>
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<td>1.05 ± 0.3</td>
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<td>9.0 ± 1.2</td>
<td>1.30 ± 0.10</td>
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<td>5.8 ± 5.8</td>
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<tr>
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<td>1.2 ± 0.3</td>
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<tr>
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<td>1.8 ± 0.7</td>
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</tbody>
</table>

— = nonfused G_{xα1}; **a** = coupling factors for FPR-Gxα1 interaction can only be given approximately because FPR expression level could not be determined (Wenzel-Seifert et al., 1999).
protein-coupled β₂AR are slower in onset than responses mediated by the Gₛ-coupled β₂AR and the Gₛ-coupled FPR. The different kinetics of receptor-G-protein interaction could thus generate intracellular signals in a timely, ordered fashion.

**GTPγS Saturation Binding to β₂AR-Gₛₐ₃L and β₂AR-Gₛₐ₅L:** The β₂AR Bound to Inverse Agonist May Actively Reduce the Apparent GTPγS Affinity of Gₛₐ₅L. Figure 4A shows a typical GTPγS saturation experiment for β₂AR-Gₛₐ₃L, and Table 1 provides a summary of the GTPγS saturation binding experiments for all fusion proteins studied. Because of the high constitutive activity of the β₂AR coupled to Gₛₐ₃L (see Fig. 3A) (Seifert et al., 1998b), GTPγS saturation binding studies for β₂AR-Gₛₐ₃L were performed in the presence of ISO and ICI. Moreover, reactions were conducted for 45 min only to detect both agonist and inverse agonist effects on GTPγS binding. We are aware of the fact that after an incubation time of 45 min, an equilibrium of the binding reaction is not yet reached, but at later time points, it becomes increasingly difficult to analyze the effect of an agonist on GTPγS binding to Gₛₐ₅L. The apparent Kᵅ values of ISO-stimulated GTPγS binding to β₂AR-Gₛₐ₃L and β₂AR-Gₛₐ₅L were in the subnanomolar range (0.4–0.7 nM). ICI reduced the apparent affinity of Gₛₐ₅L for GTPγS by about 10-fold (apparent Kᵅ, 4.2 nM). For both β₂AR-Gₛₐ₅L and β₂AR-Gₛₐ₃L, the coupling factor was ~1, indicating that the fused β₂AR interacts efficiently with its fused Gₛ partner.

To address the question whether the differential regulation by agonist and inverse agonist of the apparent GTPγS affinity of Gₛₐ₅L was an artifact induced by the specific incubation time chosen (45 min), we also determined the apparent Kᵅ values for GTPγS after a 3-h incubation, i.e., when the
binding reaction had reached a plateau. As already indicated above, ISO did not stimulate GTPγS binding to G_{as}, under these conditions but ICI was still inhibitory (apparent $K_d$ value of GTPγS binding, 4.9 ± 1.0 nM, mean ± S.D., $n = 3$). The apparent $K_d$ value for ISO-stimulated GTPγS binding to G_{as} after a 3-h incubation was 0.5 ± 0.4 nM (mean ± S.D., $n = 3$). These values compare very favorably with the values obtained after a 45-min incubation (Table 1) and show that ICI increases the $K_v$ value of GTPγS binding to G_{as} by the β2AR bound to agonist and inverse agonist are observed at short and long incubation times. The differential regulation of the apparent GTPγS affinity of G_{as} by the β2AR bound to inverse agonist and agonist is intriguing because it indicates that the β2AR bound to an inverse agonist actively reduces the apparent GTPγS affinity of G_{as}.

The conclusion that the β2AR bound to ICI actively regulates nucleotide affinities of G_{as} is supported by the findings that ICI increases the $K_v$ value of the steady-state GTPase of β2AR-G_{as} (Seifert et al., 1998a) and that ICI stimulates the binding of xanthosine 5′-triphosphate to G_{as} (Seifert et al., 1999a). Thus, our data suggest that the inverse agonist stabilizes a specific conformation in the β2AR that actively regulates nucleotide-affinities of G_{as}-proteins. Evidence for the existence of specific active states of receptors bound to inverse agonists was also obtained for cannabinoid receptors (Bouaboula et al., 1997, 1999).

**Partial Agonists Reduce the Apparent GDP Affinity of G_{as} Less Efficiently Than Full Agonists.** The differential regulation of the apparent GTPγS affinity of G_{as} by inverse agonists and full agonists raised the intriguing question of whether partial agonists increase the apparent GTPγS affinity of G_{as} to a lesser extent than a full agonist. To address the question, we determined the apparent $K_d$ value of GTPγS binding to G_{as} after a 3-h incubation, using the partial agonist dobutamine (DOB). The apparent $K_d$ value of DOB-stimulated GTPγS binding to G_{as} was 0.7 ± 0.5 nM (mean ± SD, $n = 3$) and not significantly different from the apparent $K_d$ value obtained for ISO (0.5 ± 0.4 nM). These data show that full and partial agonists do not differ from each other in their ability to alter the apparent GTPγS affinity of G_{as}.

In agreement with our data, partial agonists of the β2-adrenoceptor also do not differ from full agonists with respect to the $K_v$ of high-affinity GTP hydrolysis, i.e., there is no differential regulation of GTP affinity of G-proteins by full versus partial agonists (Wise et al., 1997).

To identify differences in the regulation of GTPγS binding...
stimulated by full and partial agonists, we studied the effects of DOB and DCI (Seifert et al., 1998b) on the time course of GTP\(\gamma\)S binding to \(\beta_2\)AR-G\(\alpha_o\)S. The rank order of efficacy of ligands at decreasing \(t_{1/2}\) of GTP\(\gamma\)S binding to G\(\alpha_o\) and at saturating G\(\alpha_o\) with GTP\(\gamma\)S was ISO > DOB > DCI (Fig. 5A). Our data clearly show that partial agonists promote guanine nucleotide exchange less efficiently than full agonists, but differential effects of ligands on the apparent GTP\(\gamma\)S affinity of G\(\alpha_o\) do not explain these differences.

A recent study of cannabinoid receptors demonstrated that a major difference between full and partial agonists is that partial agonists decrease the apparent GDP affinity of G-proteins less efficiently than full agonists (Breivogel et al., 1998). In fact, with increasing GDP concentrations, the efficacy of partial agonists at promoting GTP\(\gamma\)S binding decreases relative to the efficacy of a full agonist. Using a very similar experimental protocol as reported for cannabinoid receptors (Breivogel et al., 1998), we found that the efficacy of the partial agonists DOB and DCI at promoting GTP\(\gamma\)S binding to G\(\alpha_o\) was decreased with increasing GDP concentration (Fig. 5B). These data support the concept that agonist efficacy is related to the ability of ligands to reduce the apparent GDP affinity of G\(\alpha_o\).

**Differential Regulation of the Apparent GTP\(\gamma\)S Affinity of G-Proteins by Receptors.** The \(K_d\) value of GTP\(\gamma\)S binding to purified G\(\alpha_o\) is \(\sim 175 \text{ to } 1750\) higher (0.7 \(\mu\)M) (Northup et al., 1982) than the apparent \(K_d\) values of GTP\(\gamma\)S binding to receptor-coupled G\(\alpha_o\) (0.4–4.2 nM). These data suggest that a receptor can dramatically increase the GTP\(\gamma\)S affinity of G\(\alpha_o\). By analogy, the \(K_d\) value for GTP\(\gamma\)S binding to purified G\(\alpha_i\)-proteins is \(\sim 50 \text{ to } 100\) nM (Cartay et al., 1990), whereas the apparent \(K_d\) value for FPR-regulated GTP\(\gamma\)S binding to G\(\alpha_i\)-proteins is \(\sim 0.7 \text{ to } 1.8\) nM (Table 1) (Wenzel-Seifert et al., 1998a, 1999). In addition, the \(\beta_2\)AR substantially increases the apparent affinity of G\(\alpha_o\) for GTP\(\gamma\)S compared with purified G\(\alpha_o\) (Table 1) (Hepler et al., 1993; Chidiac et al., 1999). Thus, the data obtained for various receptors and classes of G-proteins suggest that receptors can induce a conformational change in the G\(\alpha_o\) that increases the affinity of the G-protein for GTP\(\gamma\)S (and presumably for the natural nucleotide GTP) considerably. Our data are in agreement with the concept that GTP/GTP\(\gamma\)S binding does not passively follow GDP release but that receptors actively promote GTP\(\gamma\)S binding to G-proteins (Iiri et al., 1998). Another factor that can contribute to the large differences in apparent GTP\(\gamma\)S affinities in various systems is that purified G-proteins and G-proteins in membranes can exhibit quite different properties (Gierschik et al., 1991).

The above-discussed data raise the question whether different receptors coupled to the same G-protein alter its apparent GTP\(\gamma\)S affinity in the same way. To address this question, we compared coupling of the \(\beta_2\)AR and the FPR, a prototypical G\(\alpha_i\)-protein-coupled receptor (Wenzel-Seifert et al., 1998a, 1999), to fused and nonfused G\(\alpha_o\). The apparent \(K_d\) value of FPR agonist-stimulated GTP\(\gamma\)S binding to fused and nonfused G\(\alpha_o\) is \(\sim 1\) nM (Table 1). The agonist-occupied \(\beta_2\)AR also catalyzed GTP\(\gamma\)S binding to fused and nonfused G\(\alpha_o\), but the apparent \(K_d\) values of agonist-stimulated GTP\(\gamma\)S binding to G\(\alpha_o\) were \(\sim 25 \text{ to } 70\) times higher for the \(\beta_2\)AR than for the FPR (Fig. 4B and Table 1). The apparent \(K_d\) values of ISO-stimulated GTP\(\gamma\)S binding to \(\beta_2\)AR-G\(\alpha_i\) fusion proteins are similar to the GTP\(\gamma\)S affinity of purified G\(\alpha_i\)-proteins (Table 1) (50–100 nM) (Cartay et al., 1990). These data suggest that the FPR efficiently increases the GTP\(\gamma\)S affinity of G\(\alpha_i\)-proteins, whereas the \(\beta_2\)AR does not. Thus, our data raise the intriguing hypothesis that receptor-specific G-protein conformational states exist that differ from each other in their GTP\(\gamma\)S affinity. The molecular basis for such a receptor memory of G-proteins could be differences in the G-protein-coupling domains of various receptors. In fact, the G-protein-coupling domains of the \(\beta_2\)AR and FPR are quite different (Kobilka, 1992; Miettinen et al., 1999). It is unknown why various receptors differ from each other with respect to regulation of the apparent GTP\(\gamma\)S/GTP affinity of a given G-protein. One might assume that, because of the high intracellular GTP concentration (~50 \(\mu\)M) (Otero, 1990), GTP can readily saturate all G-proteins, even if they are in a state of low GTP affinity. There is, however, evidence for constrained access of GTP to G\(\alpha_o\) in native membrane systems (Wieland and Jakobs, 1992; Klinker et al., 1994). Thus, it is possible that in vivo the GTP affinity of G-proteins critically determines their efficiency as signal transducers.
Stoichiometry of β2AR/Gi-Protein Coupling. Inefficient β2AR-induced increase in the apparent GTPγS affinity of G-proteins does not imply that the β2AR is inefficient at activating G-proteins. Indeed, the coupling factor in β2AR-Gi_{16a} and β2AR-Gi_{16b} was ~1, indicating that all β2AR molecules activated their fused Gi \_partner (Table 1).

One nonfused FPR molecule activates ~1.0 to 1.5 G\_proteins, i.e., there is rather linear signal transfer from the receptor to the G-protein (Wenzel-Seifert et al., 1999). These findings raise the question about the number of G\_proteins activated by the nonfused β2AR. The stoichiometry of receptor to G\_proteins was ~1:100 for both the β2AR- and FPR-Sf9 cell coexpression systems (Fig. 1B) (Seifert et al., 1998a; Wenzel-Seifert et al., 1998a, 1999). This stoichiometry reflects the in vivo expression stoichiometry of signaling components (Ransnä\_s and Insel, 1988; Gierschik et al., 1991; Seifert et al., 1998b; Wenzel-Seifert et al., 1998a, 1999). Of interest, one nonfused β2AR molecule activated approximately one G\_protein molecule (Table 1). These data show that the nonfused β2AR and FPR activate a similar number of G\_proteins, i.e., linear G\_protein activation is not restricted to the FPR. Additionally, our data on β2AR/Gi-protein coupling support the recent conclusion that the 1:1 stoichiometry of receptor and G-protein in fusion proteins reflects the in vivo stoichiometry of receptor-G-protein coupling more closely than was previously assumed (Seifert et al., 1999c; Milligan, 2000). These results also underline the usefulness and relevance of fusion proteins as systems to analyze receptor-G-protein coupling.

Incomplete GTPγS Saturation Binding to β2AR-Gi_{16a} Fusion Proteins Can Be Explained by Rapid GTPγS Dissociation: Role of Guanine Nucleotide Dissociation as Mechanism of G-Protein Deactivation and Implications for Receptor/G-Protein Coupling. The coupling factor in β2AR-Gi_{16a} and particularly in β2AR-Gi_{16b} was much lower than ~1 (Table 1). An explanation for these findings could be that GTPγS dissociates from Gi\_proteins much more rapidly than from G\_i- and G\_i\_proteins. In fact, GTPγS and other GTPase-resistant guanine nucleotides can dissociate from various classes of G-proteins, including G\_q-proteins (Cassel and Selinger, 1977; Higashijima et al., 1990; Bernstein et al., 1992; Kupprian et al., 1993; Breivogel et al., 1998; Chidiac et al., 1999). To address this issue, fusion proteins were loaded with [35S]GTPγS for 1 h in the absence of ISO to avoid interference with agonist-induced dissociation. [35S]GTPγS dissociation was then stimulated by the addition of unlabeled GTP\_S at a large molar excess in the absence or presence of ISO to reaction mixtures. Using this protocol, we could clearly detect time-dependent basal [35S]GTPγS dissociation in membranes expressing β2AR-Gi_{16a}, β2AR-Gi_{16b}, and β2AR-Gi_{16c} (Fig. 6). Of interest, basal [35S]GTPγS dissociation proceeded about three times faster in membranes expressing β2AR-Gi_{16a} and β2AR-Gi_{16b} (Fig. 6, B and C) than in membranes expressing β2AR-Gi_{16c} (Fig. 6A). We did not observe any effect of ISO on [35S]GTPγS dissociation in membranes expressing β2AR-Gi_{16a} and β2AR-Gi_{16b}, presumably because the basal GTPγS dissociation rate from these fusion proteins is already high. These data support the notion that rapid dissociation of [35S]GTPγS from β2AR-Gi_{16a} and β2AR-Gi_{16b} prevents these G-proteins from binding GTPγS in stoichiometric amounts.

It is generally assumed that GTPγS binding to G-proteins is quasi-irreversible (Gilman, 1987). Thus, on first glance, it may seem most unexpected that ISO decreased the \_1/2 of GTPγS dissociation from β2AR-Gi_{16a} by about 6-fold. Intriguingly, in very early studies it had already been observed that ISO induced dissociation of [3H]guanylyl imidodiphosphate from G\_in in turkey erythrocyte membranes (Cassel and Selinger, 1977). However, those early studies were not followed up later. The findings that 1 mol of β2AR-Gi_{16a} bound 1 mol of GTPγS...
GTPγS in the GTPγS saturation binding studies and that the affinity of β2AR-coupled G\textsubscript{αs} for GTPγS is very high (Fig. 4A and Table 1) are in stark contrast to the rapid agonist-induced GTPγS dissociation. The fact that the $t_{1/2}$ of ISO-stimulated GTPγS binding was only moderately lower than the $t_{1/2}$ of ISO-stimulated GTPγS dissociation suggests that GTPγS dissociation occurs already in the initial phase of the GTPγS association experiment and, therefore, delays net GTPγS association.

The GTPγS dissociation studies bear important implications for the mechanism by which G-proteins are deactivated. It is generally accepted that the hydrolysis of GTP to GDP and inorganic phosphate determines the transition of the G-protein from the active to the inactive state (Gilman, 1987; Iiri et al., 1998). However, in a recent study we observed dissociations in the efficacies of β2AR agonists at supporting adenylyl cyclase activation in the presence of inosine 5′-triphosphate and their efficacy at hydrolyzing inosine 5′-triphosphate (Seifert et al., 1999a). In addition, xanthosine 5′-triphosphate supports β2AR-mediated adenylyl cyclase activation, but xanthosine 5′-triphosphate is not hydrolyzed (Seifert et al., 1999a). Taken together, all these data indicate that nucleotide dissociation is an important mechanism of G-protein deactivation.

The observation of highly efficient β2AR-stimulated GTPγS dissociation from G\textsubscript{αs} helps us understand the physiological interaction of receptors and G-proteins. These data suggest that G\textsubscript{αs} bound to GTPγS is in physical contact with the β2AR, despite the reduction of agonist affinity of the β2AR by GTPγS (Figs. 2 and 6). Evidence for continuous physical contact between receptor and G-protein during the entire G-protein cycle was already obtained for muscarinic acetylcholine receptors (Matesic et al., 1989). GTPγS-insensitive ternary complex formation also argues for contact of the β2AR with its G-protein partner during the entire G-protein cycle (see Fig. 2) (Széle and Fritchett, 1993; Gürdal et al., 1997; Seifert et al., 1998a). Thus, in contrast to the generally held opinion (Gilman, 1987; Iiri et al., 1998), G-protein deactivation may be a step in the G-protein cycle that is under the direct control of the receptor. Evidence for direct regulation of nucleoside 5′-triphosphate dissociation and hydrolysis of G\textsubscript{αs} by the β2AR was also provided by two previous studies from our group (Wenzel-Seifert et al., 1998b; Seifert et al., 1999b).

**Pharmacological Profile of β2AR-G\textsubscript{αs} Fusion Proteins: Evidence for Ligand-Specific Conformations of the β2AR with Different G-Protein Coupling.** It has been shown that in some systems the pharmacological profile of a receptor depends on the specific G-protein to which the receptor is coupled (Eason et al., 1994; Gettys et al., 1994; Gürwitz et al., 1994). These findings can be interpreted to mean that specific ligands stabilize ligand-specific receptor conformations that differ from each other in their ability to activate different G-proteins. To address this hypothesis, we determined the effects of the β2AR agonists ISO, SAL, DOB, EPH, and DCI and the inverse agonist ICI on GTPγS binding to β2AR-G\textsubscript{αs} fusion proteins. We assessed both ligand efficacies and ligand potencies.

Efficacies of ligands at β2AR-G\textsubscript{αs} fusion proteins were analyzed in two ways (Table 2). First, we analyzed the efficacies of a given ligand at the various fusion proteins. Second, we analyzed the rank order of efficacies of agonists at the different fusion proteins. The efficacies of a given ligand varied largely at the different fusion proteins (0.55–1.03 for SAL; 0.40–0.98 for DOB; 0.26–0.98 for EPH; 0.08–0.67 for DCI and −0.15–0.00 for ICI). The efficacies of SAL and EPH at β2AR-G\textsubscript{αq} were considerably higher than at β2AR-G\textsubscript{αs}, β2AR-G\textsubscript{αi}, and β2AR-G\textsubscript{αq} whereas the efficacy of DCI was lower at β2AR-G\textsubscript{αq} than at β2AR-G\textsubscript{αs}, β2AR-G\textsubscript{αi}, and β2AR-G\textsubscript{αq}. Moreover, the rank order of efficacy of ligands at activating GTPγS binding to β2AR-G\textsubscript{αq} was ISO ≫ SAL > DOB > EPH > DCI, whereas the rank order of efficacy at β2AR-G\textsubscript{αs} was ISO ≫ SAL > DOB > EPH > DCI. We also observed

**Table 2**

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<th>Construct</th>
<th>ISO</th>
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<th>EPH</th>
<th>DCI</th>
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<td>0.29 ± 0.07</td>
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<tr>
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**Table 3**

Potencies of β2AR ligands at stimulating GTPγS binding to β2AR-G\textsubscript{αs} fusion proteins

For determination of the potencies of ligands, GTPγS binding was measured in Sf9 membranes expressing β2AR-G\textsubscript{αs} at 3.7 to 13.0 nM pmol/mg with 0.4 nM [35S]GTPγS as described under Experimental Procedures. The incubation time was 45 min. Reaction mixtures contained ligands at 0.1 nM to 1 nM as appropriate to obtain saturated concentration-response curves. The EC\textsubscript{50} values are given in nM and were calculated by nonlinear regression. Data shown are the means ± S.D. values of three independent experiments performed in triplicates.

<table>
<thead>
<tr>
<th>Construct</th>
<th>ISO</th>
<th>SAL</th>
<th>DOB</th>
<th>EPH</th>
<th>DCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2AR-G\textsubscript{αs}</td>
<td>1.7 ± 0.5</td>
<td>39 ± 23</td>
<td>90 ± 16</td>
<td>923 ± 270</td>
<td>13 ± 12</td>
</tr>
<tr>
<td>β2AR-G\textsubscript{αs}</td>
<td>6.2 ± 3.3</td>
<td>167 ± 78</td>
<td>310 ± 108</td>
<td>3600 ± 498</td>
<td>36 ± 15</td>
</tr>
<tr>
<td>β2AR-G\textsubscript{αi}</td>
<td>31 ± 16</td>
<td>712 ± 401</td>
<td>1860 ± 890</td>
<td>1850 ± 500</td>
<td>15 ± 8</td>
</tr>
<tr>
<td>β2AR-G\textsubscript{αq}</td>
<td>42 ± 18</td>
<td>809 ± 241</td>
<td>155 ± 90</td>
<td>917 ± 10</td>
<td>—</td>
</tr>
<tr>
<td>β2AR-G\textsubscript{αq}</td>
<td>143 ± 69</td>
<td>537 ± 311</td>
<td>595 ± 109</td>
<td>5990 ± 2130</td>
<td>—</td>
</tr>
<tr>
<td>β2AR-G\textsubscript{αq}</td>
<td>23 ± 13</td>
<td>310 ± 132</td>
<td>540 ± 211</td>
<td>4670 ± 1110</td>
<td>—</td>
</tr>
</tbody>
</table>

*—, DCI-stimulated GTPγS binding was too small to calculate an EC\textsubscript{50} value.*
differences in agonist efficacies of the β2AR coupled to G\textsubscript{T}\textsubscript{q} proteins. Specifically, at β2AR-G\textsubscript{16α}, ligands activated GTP\textsubscript{yS} binding in the order of efficacy ISO > SAL > DOB ≈ EPH > DCI. In contrast, the order of efficacy of ligands at β2AR-G\textsubscript{16α}, was ISO ≈ SAL > DOB > EPH > DCI. These data show that the efficacies of typical β2AR ligands differ from each other, depending on to which G-protein the receptor is coupled. There are even differences in the pharmacological profile of the β2AR coupled to different members of the same G-protein family, be it G\textsubscript{s}, G\textsubscript{i}, or G\textsubscript{T}\textsubscript{q}.

Differences in the pharmacological profile of the β2AR coupled to different G-proteins were also evident upon analysis of ligand potencies (Table 3). We observed large variations in the potencies of a given ligand at stimulating GTP\textsubscript{yS} binding to the different fusion proteins. The variation of the EC\textsubscript{50} values was 1.7 to 143 nM for ISO, 39 to 809 nM for SAL, 90 to 1860 nM for DOB, and 917 to 5990 nM for EPH. As was observed for agonist efficacies, the potencies of ligands did not vary systematically for different G-proteins. For example, at β2AR-G\textsubscript{16α}, ISO and EPH exhibited a particularly low potency, whereas at β2AR-G\textsubscript{16ω}, DOB showed a very low potency. We also observed variations in the rank order of potency of ligands at the different fusion proteins. At β2AR-G\textsubscript{16α} fusion proteins, the rank order of potency was ISO > SAL > DOB > EPH. Intriguingly, the rank order of potency of ligands at β2AR-G\textsubscript{16ω} was different from the corresponding rank order at β2AR-G\textsubscript{16ω} (ISO > DOB > SAL) (Table 3). Taken together, our data clearly show that the pharmacological properties of the β2AR depend on to what G-protein the β2AR is coupled. These data are compatible with a model in which ligand-specific receptor conformations exist. Those ligand-specific receptor conformations differ from each other in their ability to activate different G-proteins. Our conclusions for the β2AR are supported by data regarding the pharmacological profiles of the 5-hydroxytryptamine\textsubscript{1A} receptor, α\textsubscript{2}-adrenoceptor, and muscarinic acetylcholine receptors coupled to different G-proteins (Eason et al., 1994; Gettys et al., 1994; Gurwitz et al., 1994).

Pertussis toxin uncouples agonist-free and agonist-occupied receptors from G\textsubscript{i} proteins (Gierschik et al., 1991; Wenzel-Seifert et al., 1989a). By analyzing the effect of pertussis toxin on the coupling of the β2AR to G\textsubscript{i} proteins in cardiac myocytes, Xiao et al. (1999) concluded that, in this coupling situation, the β2AR is not constitutively active. At β2AR-G\textsubscript{16ω} and β2AR-G\textsubscript{16α}, the efficacies and potencies of partial agonists were, in general, considerably smaller than at β2AR-G\textsubscript{16α} (Tables 1 and 2). These findings corroborate the conclusion by Xiao et al. (1999) that the G\textsubscript{i}-protein-coupled β2AR is not constitutively active.

The analysis of the effects of agonists and inverse agonists at β2AR-G\textsubscript{16ω} did also not provide evidence for constitutive activity of the β2AR in this coupling setting (Tables 2 and 3). For β2AR-G\textsubscript{16ω}, we observed an unexpectedly high efficacy (but not potency) of SAL at activating GTP\textsubscript{yS} binding. However, this finding does not allow the conclusion that the β2AR coupled to G\textsubscript{i} is constitutively active, because the analysis of the effects of partial agonists and inverse agonists did not reveal additional evidence for constitutive activity of the β2AR in this coupling situation.
Eason MG, Jacinto MT and Liggett SB (1994) Contribution of ligand structure to activation of \( \alpha_2 \)-adrenergic receptor subtype coupling to \( G_s \). Mol Pharmacol 45: 696–702.


Hepler JR, Kozasa T, Smreczak Y, Simon MJ, Rhee SG, Sternweis PC and Gilman AG (1994) Mastoparan may activate GTP hydrolysis by Gi-proteins in HL-60 membranes: Evidence that the chemotactic peptide receptor to phospholipase C.


